Application of a 16S rRNA PCR/HRMA Assay for Rapid Detection of Salmonella Bacteremia: A Case Report

Kevin Jeng1,2*, Samuel Yang1, Helen Won1, Charlotte Gaydos1, Yu-Hsiang Hsieh1, Alex Kecojevic1, Karen Carroll1, Justin Hardick1, Richard Eric Rothman1

Johns Hopkins University, Baltimore, MD, USA1, Duke University, Durham, NC, USA2

* Corresponding author. Mailing address: 5801 Smith Avenue, Suite 3220, Davis Building, Baltimore, MD 21209. Phone: 443-287-5403. Fax: 410-735-6440. Email: kyj2@duke.edu

Abstract

Current culture and phenotypic protocols for diagnosing Salmonella infections can be time consuming. Here, we describe the application of a 16S rRNA PCR coupled to high resolution melt analysis for species and serotype identification in six hours of blood sample collection from a patient with Salmonella serotype Enteritidis bacteremia.

Case Report

A man in his 40s was transferred from an outpatient clinic to the Johns Hopkins Emergency Department (ED) after complaining of fevers on hospital day 0 for evaluation of suspected bacteremia. Aerobic and anaerobic blood cultures were drawn and sent to the Johns Hopkins Hospital central laboratory from the outpatient clinic, and the patient was given 1 dose of intravenous vancomycin and cefoxitin for suspected bacteremia prior to ED presentation. In the ED, the patient’s temperature was 37.9°C, and blood was drawn for routine admission.
laboratory testing. The patient’s white blood cell count was 4300 cells/μl with 82% polymorphonuclear leukocytes and 7% lymphocytes, and the patient was admitted for further evaluation. An extra 5 ml blood sample was collected in EDTA tubes (Becton-Dickinson, Franklin Lakes, NJ, USA) upon presentation to the ED for an ongoing study evaluating molecular diagnostic assays wherein patients with suspected bacteremia were prospectively asked to donate blood samples for research and provide permission for researchers to review basic de-identified information regarding presenting complaint, laboratory data, antibiotic use, and clinical outcome.

The clinical blood culture bottles were processed in the hospital laboratory using the BacT/ALERT 3-D blood culture system (BioMérieux, Durham NC). Gram negative bacilli grew in one set of bottles 17.5 hours after collection, and antimicrobial therapy was switched to piperacillin/tazobactam. The bacterial isolate was subcultured onto standard laboratory media, and a pan-susceptible *Salmonella*, serogroup D, was identified on hospital day 3 using the Phoenix Microbial identification system (Becton-Dickinson, Sparks, MD) followed by confirmation using serogroup specific agglutination reagents. Antibiotics were further switched to ampicillin and the patient was discharged the next day. An isolate was sent to the Maryland State Health Laboratory for conventional serotyping, and *Salmonella* serotype Enteritidis was definitively identified 11 days later. The 3 blood cultures drawn after hospital day 1 were all negative for bacterial growth.

A stool sample was collected on hospital day 1. On hospital day 3, the stool sample also became positive by culture for pan-susceptible *Salmonella*. It was sent to the Maryland State Health Department for identification and typing and found to be *Salmonella* serotype Enteritidis as well.
The 5 ml whole blood sample collected upon presentation in the ED for research purposes was taken to the research laboratory, where it was incubated for 3 hours at 37°C with 3 mL trypsinase soy broth (Becton-Dickinson, Baltimore, MD). 3 hours was determined in previous optimization studies as a preincubation period that maximized organism detection from blood without compromising time to results. After incubation, bacterial DNA was extracted and processed with a nucleic acid amplification assay that exploits the bacterial-specific 16S rRNA gene for rapid pathogen identification, as previously described (16, 18). Briefly, PCR analysis was performed in a 10 μl reaction comprising 8 μl PCR master mix (Idaho Technology, Salt Lake City), and 2 μl target input. Cycling conditions were performed as previously described (18). This assay uses multiple primer pairs (Table 1) that target three hypervariable regions in the 16S rRNA gene (V1, V3 and V6) to generate a diverse range of species-specific amplicons. These amplicons can be differentiated using high-resolution melt analysis (HRMA), which records variations in amplicon melting patterns (16). Using this method, we have generated a melt curve library of 60 common species of bacteria associated with bacteremia, including 9 *Salmonella* serotypes, both typhoidal and nontyphoidal.

In the blood sample collected from this patient, analysis with HRMA using the V1, V3, and V6 primers flanking three hypervariable regions within the 16S rRNA gene identified the presence of *Salmonella* serotype Enteritidis (Figures 1a-1b, region V1 not shown). Total assay time was approximately 6 hours after sample receipt (3 hours incubation, 2 hours DNA extraction, 1 hour PCR amplification, 5 minutes HRMA). This PCR assay was not performed in real time, as it was part of a research protocol. The extracted bacterial DNA was stored at -20°C and processed by PCR a week after the patient’s initial presentation.
Non-typhoidal human salmonellosis is a significant food-borne infection with an estimated 1.4 million cases each year in the United States, of which 40,000 are confirmed by culture and reported to the CDC (15). *Salmonella* infection presents with variable severity—from mild, limited gastroenteritis to invasive disease involving bacteremia and localized extragastrointestinal infections (5). Timely and cost-effective *Salmonella* diagnosis and typing is important for clinical purposes, as early identification of the pathogen helps in directing therapy (17). The current standard protocol for diagnosing salmonellosis, via microbiological culture at an on-site laboratory followed by serotyping at a public health laboratory, is slow and complicated due to sample transport and required culture incubation time. In addition, administration of antibiotics prior to blood culture collection may lead to false negative results, further hindering clinical diagnosis.

Previous studies have investigated the utility of *Salmonella*–specific PCR assays in diagnosing salmonellosis from blood samples (1, 2, 4, 6, 7, 10, 11). This case was different in that it utilized a broad range PCR assay that incorporated detection of various *Salmonella* serovars into a general diagnostic library of 60 eubacteria for rapid diagnosis of bacteremia. With conventional methods, a serogroup was reported 3 days after the patient’s initial presentation, and finalized results were not confirmed until 11 days after. In contrast, our PCR/HRMA assay could have identified the sample as *Salmonella* serotype Enteritidis within 6 hours of blood sample collection. Furthermore, changes in antimicrobial treatment occurred with each piece of incremental culture data from the microbiology laboratory, demonstrating the importance of timely microbiological data on clinical decision making. Previous literature has established that faster time to Gram stain reporting is independently associated with shorter
lengths of hospital stay in blood stream infections (3), and plays a significant role in modifying empiric therapy (13, 14). While bacterial susceptibility data is critical to patient care, a 2003 study by Munson et al. showed that notification of Gram stain results in cases of bacteremia had a significantly larger role in determining antimicrobial management than the release of antimicrobial susceptibility data (9). The rapid species-level detection that 16S rRNA PCR/HRMA provides is even more specific than the Gram stain, and can be available to the physician within 6 hours of blood sample collection. In contrast, BacT/Alert FN systems take 17-20 hours on average to detect positive blood cultures (8, 12). Thus, using the 16S rRNA PCR/HRMA assay in conjunction with standard culturing methods could provide physicians with critical microbiological data earlier in the course of treatment than is currently feasible, possibly improving the care of bacteremic patients. However, further studies are needed to establish the assay’s sensitivity, specificity, and ultimate clinical utility before integration into a diagnostic algorithm.

An added advantage of molecular diagnostic assays demonstrated by this case is that they can detect organisms even after antibiotics are administered. When a patient is septic, blood cultures must be drawn before the initiation of antimicrobial therapy, or growth in culture may be inhibited. Since 16S rRNA PCR/HRMA relies on nucleic acid detection for pathogen identification, antibiotics that inhibit microbial growth do not affect assay results. Notably, the blood sample from which Salmonella serotype Enteritidis was identified by 16S rRNA PCR/HRMA was drawn after the patient had already been started on vancomycin and cefoxitin, and all subsequent blood cultures after initiation of empiric antibiotics were negative. Molecular assays such as our 16S rRNA PCR/HRMA may serve as useful clinical adjunctive tests to provide physicians with early detection of pathogens causing bacteremia.
Further research dedicated to validating PCR assays for rapid *Salmonella* serotyping is important for addressing the mortality and morbidity associated with sepsis caused by *Salmonella* species.

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References


TABLE 1: Primers targeting the bacterial 16S rRNA gene for HRMA\(^1\) analysis.

<table>
<thead>
<tr>
<th>Region</th>
<th>Primer</th>
<th>Sequence</th>
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<tbody>
<tr>
<td>V1</td>
<td>V1-1</td>
<td>5'-GYGGCGNACGGGTGAGTAA</td>
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<tr>
<td></td>
<td>V1-2</td>
<td>5'- TTACCCCACCAACTAGC</td>
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<tr>
<td>V3</td>
<td>V3-1</td>
<td>5'- CCAGACTCCTACGGGAGGCAG</td>
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<tr>
<td></td>
<td>V3-2</td>
<td>5'- CGTATTACGCGGCTGCTG</td>
</tr>
<tr>
<td>V6</td>
<td>V6-1</td>
<td>5'- TGGAGCATGTGGTTAATTCGA</td>
</tr>
<tr>
<td></td>
<td>V6-2</td>
<td>5'- AGCTGACGACANCCATGCA</td>
</tr>
</tbody>
</table>

\(^1\) HRMA: High Resolution Melt Analysis

FIGURE 1: Comparison of V3 (FIGURE 1a) and V6 (FIGURE 1b) 16S rRNA hypervariable region melting curves of bacterial DNA isolated from the patient (solid line) versus melting curves of a *Salmonella* serotype Enteritidis control (dotted line), a *Salmonella* serotype Choleraesuis control (dotted line), and *Staphylococcus aureus* control (dotted line).

Figure 1a. Comparison of V3 Melting Curves.

Figure 1b. Comparison of V6 Melting Curves